MICROSCOPIC EVALUATION OF MYONECROSIS INDUCED IN MURINE SKELETAL MUSCLE BY *SISTRURUS MILIARUS BARBOURI* (DUSKY PYGMY RATTLESNAKE) CRUDE VENOM

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CHAPTER I

Introduction and Literature Review

Venomous snakes have been classified into four families that contain some of the most dangerous animals on the Earth. These families are: 1) Elapidae, 2) Hydrophiidea, 3) Colubridae and 4) Viperidae. The Elapidae family contains probably the best known venomous snakes: the Indian Cobra (*Naja naja naja*) which is known for it's unique facelike marking on the dorsum of the hood, the King Cobra (*Ophiophagus hannah*), the kraits (the *Bungarus* genus), the spitting cobras (*Hemachatus*), the Coral snakes (*Micrurus* genus) and others (Brown, 1973). The Hydrophiidea Family includes the sea snakes and is composed of several genera. These snakes have vertically flattened tails that they use like paddles. Most species stay near to the shores of most major oceans in the temperate and tropical regions of the world. One species, however, *Pelamis platurus* is truly oceanic (Brown, 1973). The Colubrid snakes are best represented by the Boomslang, *Dispholidus typus,* which is an arboreal snake found in the rain forests of Africa. They are opisthoglyphic snakes meaning that they have small fangs in the back of the upper jaw and therefore must chew their prey to envenomate them. The Viperidae includes the puff adders, vipers and pit vipers of subfamily Crotalinae. Named for the fact that they birth live young, the family Viperidae contains about 16 genera and 144 recognized species (Campbell and Brodie, 1992). Some of the better known

Viperids are the puff adders of the genus *Bitis* (named for their habit of puffing up and hissing when approached) , the only venomous snake found in Great Britain, *Vipera berus* (the common viper), and the rattlesnakes (Brown, 1973).

The rattlesnakes belong to a subfamily of the Viperidae family, Crotalinae, with the massasaugas, cottonmouths, copperheads, bushmaster and other pit vipers (Campbell and Brodie, 1992). As suggested by the name "pit viper", these snakes all possess pits just below the eyes that contain organs extremely sensitive to heat. Of the pit vipers, the only members known to posses actual rattles made from specialized scales are included in two genera, *Crotalus* and *Sistrurus*. There are, however, some species within the *Crotalus* genus that characteristically lack rattles altogether, namely *Crotalus catalinensis* and *C. ruber lorenzoensis* (Glenn and Straight, 1982). More than 30 species and seventy subspecies of rattlesnake are recognized in the world today (Klauber, 1982) and all are considered to be venomous. The genera *Crotalus, Sistrurus* and *Agkistrodon* are the only native members of of the Crotalidae in the United States. Table 1 summarizes the composition of the subfamily Crotalinae.

Several distinct morphological differences can be noted between the members of the *Crotalus* and *Sistrurus* genera. Most obvious of the differences is the generally smaller size of the so-called pygmy rattlesnakes of the genus *Sistrurus* as opposed to the larger individuals of the *Crotalus* genus. Another distinguishing characteristic between the two genera is the organization of crown

Table 1: Summary of the Members of the Subfamily Crotalidae

Genus	Common Name
Crotalus	Rattlesnakes
Sistrurus	Massasaugas and pigmy rattlesnakes
Lachesis	Bushmaster
Bothrops	New World pit vipers
Trimeresurus	Asiatic pit vipers
Agkistrodon	Moccasins and copperhead

scales or large plate-like scales found on the head. Members of the *Sistrurus* genus have a characteristic group of 9 large plates on the dorsal region of the head (including the two supraorbital plates) whereas the members of the *Crotalus* genus have many more numerous and smaller plates in this area (Glenn and Straight, 1982).

Another major difference between the two rattlesnake genera is their geographic distribution. The genus *Crotalus* contains individuals that are widely scattered across the Western Hemisphere (North, Central and South America) whereas members of the *Sistrurus* genus are limited to North America. There are three recognized species of *Sistrurus*: *S. catenatus*, *S. miliarus* (these two are found in the United States primarily) and *S. ravus* (located in the Southern part of the Mexican plateau (Gans, 1978). The recognized subspecies are: *S. catenatus catenatus* (Eastern massasauga), *S. c. edwardsii* (dessert massasauga), *S. c. tergeminus* (Western massasauga), *S. miliarus miliarus* (Carolina pygmy rattlesnake), *S. m. barbouri* (Eastern pygmy rattlesnake), *S. m. streckeri* (Western or Dusky pygmy rattlesnake), *S. ravus ravus* (Mexican pygmy rattlesnake), *S. r. brunneus* (Oaxacan pygmy rattlesnake) and *S. r. exiguus* (Guerreran pygmy rattlesnake) (Glenn and Straight, 1982). Although the amount of knowledge concerning rattlesnakes continues to grow, most of the advancement appears to be in the understanding of the *Crotalus* genus of snakes while the *Sistrurus* species still remains relatively unstudied. This work involves a subspecies of *Sistrurus miliarus* that is found in the Central and South regions of the United States; *S. m. barbouri* (Dusky pigmy rattlesnake).

Globally, bites from venomous snakes are of significant concern. The World Health Organization estimated in 1954 that approximately 500,000 envenomations occurred each year and that 40,000 of these were fatal (Swaroop and Grab, 1954). In the United States, however, the incidence is much lower. In fact, a study conducted between the years 1950-1959 concluded that in the USA, 188 people died due to the bites of venomous snakes; 20% of these bites were attributed to rattlesnakes (Parrish, 1980) . Parrish reported that of the estimated 45,000 snakebites reported in the United States, 7,000-8,000 were caused by venomous snakes with an estimated 12-15 fatalities per year (Parrish, 1980). Studies (Dart *et al.*, 1992) conducted by a cooperative effort between the Section of Emergency Medicine and Arizona Poison and Drug Information Center have established the Western Envenomation Database (WED). The WED included reports from 132 patients ranging in age from 0 to 79 years of age and from 24 states. These patients were followed closely for at least one month after initial treatment of the bites. Of these 132 patients three succombed to the effects of the bite and died. Two of these deaths occured in children under the age of eight years. Terribly, one of the two children died because of a bite recieved when an adult draped a rattlesnake around her neck (Dart *et al.*, 1992). In all three cases there was a serious lack of appropriate medical attention given to alleviate both the local and systemic actions of the venom (Dart *et al.*, 1992).

The major clinical manifestations of snake bites result from both systemic and local action of the venom components (Gomez and Dart, 1995).

The most pronounced systemic effects of rattlesnake venom-induced injury are hemorrhage, hypotension, shock, coagulopathies, neurotoxicity and death. The most prominent of the local effects observed with snake bites are hemorrhage, massive edema, dermonecrosis, and myonecrosis (Ownby, 1990). Sometimes blebbing, sloughing of the skin and total amputation of a limb has been observed in severe envenomations.

With such severe sequelea being produced by crotalid venoms it is understandable that the major recourse clinically has been the use of a polyvalent anti-serum produced against the crude venom. Wyeth's Polyvalent (Crotalidae) antivenom (Wyeth-Ayerst Laboratories Inc., Marrietta, PA., U.S.A.) consists of the hyperimmune serum from horses immunized with crude venom from four crotalid snake species, *Crotalus atrox, C. adamanteus, C. durissus terrificus,* and *Bothrops atrox*. These four species are used due to the fact that they contain some of the most potent of the crotalid toxins and the anitbodies produced against these venoms are highly cross-reactive with components of other venoms (Gingrich and Hohenadel, 1956). Although this antivenom has been shown to be effective at reducing lethality (Russell*et al.*, 1973), it is not nearly as efficacious in the prevention of local myonecrosis and hemorrhage induced by crotalid snake venom (Ownby *et al.*, 1983). Therefore, myonecrosis, hemorrhage, and edema continue to be of great consequence in the clinical treatment of snake bite.

There are other difficulties associated with the use of polyvalent (Crotalidae) antivenom besides low efficacy against locally active toxins.

Perhaps the most important of these is anaphylaxis. The most common cause of anaphylaxis is exposure to a foreign, usually animal, protein. Although with the development of vaccines and human immune gamma globulin therapy, the use of animal-derived products is becoming rarer in clinical practice. The two most notable exceptions are in the use of antihuman lymphocyte globulin used in some hospitals to reduce transplant rejections and the use of horse serum derived antivenom in snake bite (Jurkovich *et al.*, 1988). The presence of foreign horse protein often causes an anaphylactic reaction that occurs when the foreign protein binds to antibodies (IgE) that are bound to a mast cell. This leads to massive mast cell degranulation. Histamine, heparin and other vasoactive compounds are released in tremendous quantities and can lead to severe systemic compromise such as hypotension, bronchoconstriction, shock, bradycardia and in severe cases, total cardiovascular collapse. (Jurkovich, *et al.*, 1988). To counteract this effect the use of antihistamines like Benadryl® (diphenhydramine HCl) has been advocated, especially when combined with epinephrine which is the endogenous physiological antagonist to histamine (Jurkovich *et al.*, 1988). The use of corticosteroids has also been studied and found to be of some benefit in the prevention of serum sickness (Jurkovich *et al.*, 1988; Parrish*et al.*, 1965).

Another manifestation of hypersensitivity that is somewhat different than the anaphylactic response just discussed and much more common (up to 50% of patients) is serum sickness. This syndrome is characterized by fever, swollen lymph nodes, generalized urticarial rash and painful joints that result from the

deposition of antigen-antibody complexes in these areas. It is a Type III hypersensitivity reaction that tends to occur around one week to ten days after treatment with antivenom. Patients with serum sickness have also responded to the use of antihistamines and steroid treatments (Jurkovich *et al.*, 1988). A final serious drawback to the use of antivenom therapy is the great cost. In 1988, Jurkovich *et al.*, stated that one vial of Wyeth's Polyvalent (Crotalidae) antivenom (10 ml.) cost $100.00. When the average amount of antivenom he reported used in an envenomation was 20 vials (with a range of 1-118 vials depending upon clinical severity of the bite) the cost becomes enormous. Currently, one vial of Wyeth's Polyvalent (Crotalidae) Antivenom costs $130.00. The use of antivenom does have some merit, but current therapies must be revised to better determine the need for antivenom therapy and more efficacious antivenom formulations must be devised to increase the benefit and decrease the cost.

Because of the ineffective neutralization of hemorrhagic and myotoxic properties of the venom by commercial antivenom much effort has been expended to isolate individual toxic components from the crude venom. In theory, isolation of these toxins and production of antibodies specific for these isolated compounds should yield an antivenom that is more efficacious against the actions of the isolated component. In fact, Ownby *et al.* (1983) showed that antivenom prepared to a pure myotoxin isolated from *Crotalus viridis viridis* (Prairie rattlesnake) is more effective in neutralizing myonecrosis induced by this toxin and crude venom than is Wyeth's antivenom. Because of these findings,

recent work has been aimed at the isolation, purification and characterization of individual toxins. After isolation, these toxins must be assayed for their biological activity such as causing hemorrhage (hemorrhagic toxins), edema (edema forming toxins) or muscle cell necrosis (myotoxins). This work has led to the isolation and identification of many myotoxins.

The action of isolated polypeptide snake-venom myotoxins on muscle after injection *in vivo* has been studied at both the light and electron microscopic levels (Ownby, 1990). These studies have revealed that there are several types of toxins present in crude venoms that act in concert to produce the final toxic effects of the venom. The necrosis seen after injection of a crude venom may be due either to these components singly or to the interaction of several of them culminating in the lesions observed. Ownby and Colberg (1988) stated that there is a time at which all the cells that have been damaged by different toxins reach a common stage or appearance which may correspond to the final necrotic state. Therefore, in elucidating the mechanisms by which snake venoms act, it has become necessary to isolate and characterize purified toxic components.

Many of these polypeptides have been isolated in pure form and their myotoxic attributes studied in detail (Mebs and Ownby, 1990; Ownby, 1990). A large number of proteins falling into essentially three distinct categories have been isolated to date. Currently, they are described as either 1) small, basic myotoxins, 2) cardiotoxins (found in elapid venoms) and 3) phospholipase A_2 (PLA_2) myotoxins (which includes two subgroups: those PLA_2 myotoxins that are also presynaptic neurotoxins, and those PLA_2 myotoxins that are not

neurotoxic). These non-neurotoxic PLA_2 myotoxins are also subdivided into two groups: those that have and those that lack PLA_2 enzymatic activity. These compounds are structurally very similar to PLA_2 yet have been shown not to posses enzymatic activity.

When crude prairie rattlesnake venom is injected intramuscularly into mice, it produces very characteristic lesions in muscle cells. This is due in part to the action of a small, basic myotoxin called myotoxin *a*. isolated from the prairie rattlesnake, (*Crotalus viridis viridis*) (Ownby et al., 1976; Cameron and Tu, 1977). Myotoxin *a* has been well studied and serves as an excellent example of the characteristics of this group (Table 2). Chemically, myotoxin *a* is a peptide of 39 amino acid residues with a pI of 9.6 and an estimated molecular weight of 4.1kD (Cameron and Tu, 1977). It is bound tightly in a random coil formation by two disulfide bridges which appear to be necessary for the biological activity and stability of the protein (Cameron and Tu, 1977).

Histologic studies of the lesions induced by purified myotoxin *a* at both the light and electron microscopic levels reveal that it induces dilation of the sarcoplasmic reticulum and perinuclear space while leaving T-tubules intact (Ownby *et al.*, 1976). This causes a distinctive vacuolated appearance of the cells at the light microscopic level. These toxins have a specific action against muscle cells since no detectable change in morphology of adjacent endothelial cells or fibroblasts in the vicinity was seen in histologic sections (Ownby *et al.*,

Table 2: Characteristics and Sources of Small, Basic Myotoxic Compounds Isolated From Rattlesnake Venoms.

Class	Characteristics	Sources	Reference
Small, Basic Myotoxins	Basic, non-enzymatic, single chain peptides of 42-45 a.a.	1. *Crotalus viridis viridis*- myotoxin *a*	1. Cameron and Tu (1977); Ownby *et al.* (1976)
		2. *C. durissus terrificus* - crotamine	2. Laure, (1975)
		3. *C. v. helleri*- peptide c	3. Maeda *et al.*, (1978)
		4. *C. v. concolor*- myotoxin I and II	4. Engle *et al.*, (1983); Bieber *et al.*, (1987)
		5. *C. horridus horridus*- toxin III	5. Mebs *et al.*, (1983)
		6. *C. adamanteus*- CAM toxin	6. Samejima *et al.*, (1988)

1976). Electron microscopic histocytochemical studies on frozen human muscle cells have demonstrated a high affinity of peroxidase-conjugated myotoxin *a* to the membrane elements of the sarcoplasmic reticulum (Tu, 1982). However, a direct binding of the toxin to intact skeletal muscle cell membranes has yet to be definitively established .

Some work has been done *in vitro* on myoblasts in culture to attempt to elucidate direct effects of the small, basic myotoxins on these cells. However, these cells appeared to be unaffected by the application of purified toxin (Baker *et al.*, 1993 ; Brusés *et al.*, 1993).

A second group of myotoxic compounds found in snake venoms is the cardiotoxins (Ownby *et al.*, 1993). These toxins have only been isolated from the Elapidae species, especially the cobras (*Naja* genus) and the ringhal (*Hemachetus* genus), and were named for their ability to cause cardiac arrest both in the live animal and in *in vitro* studies (Harvey, 1990; Harris and Cullen 1990).

Even though these toxins are exclusive to the Elapidae venoms they are important in the present discussion of myonecrosis for two reasons. First they are membrane active toxins that cause a specific type of lesion that is separate and distinct from that induced by the small, basic myotoxins. Second, this lesion is very similar to that induced by the PLA_2 type of myotoxins (Ownby *et al.*, 1993). These similarities may reflect a similar mechanism of toxicity. This may provide valuable clues to the mechanism utilized by the PLA_2 myotoxins.

Structurally, cardiotoxins are similar to the α-bungarotoxins according to Dufton and Hider (1991), but lacking the post-synaptic neurotoxicity of these proteins. They are larger proteins of about 60-62 a.a. in length and have pIs in the basic range. These single chain peptides are folded over and secured by four disulfide bonds (Harvey, 1990).

The cardiotoxins do not posses the specificity of the small, basic myotoxins , but are generally cytotoxic molecules (Dufton and Hider, 1991; Kini and Evans, 1989 and Harvey, 1990). Ownby and Colberg, (1988) observed following the injection of crude Indian cobra venom (*Naja naja* species) extensive myonecrosis characterized by the formation of triangular-shaped "delta" lesions, as well as very tightly or densely clumped myofibrils in the cytoplasm occurred. In a subsequent investigation with cardiotoxin-1 (isolated from *Naja naja atra*, the Chinese cobra), Ownby *et al.* (1993) observed the same type of pathology suggesting that this type of pathology was indeed due to the cardiotoxin. The mechanism of toxicity for the cardiotoxins has not been fully understood, although there have been many ideas presented (Harvey, 1985 ; Dufton and Hider, 1991). All of these hypotheses center around membrane effects such as a direct "detergent-like" destruction of the membrane, aggregation of membrane bound proteins, formation of membrane channels and stimulation of endogenous PLA2 (Harvey, 1990).

The observation of delta lesion production in the myonecrosis induced by the cardiotoxins is of relevance to the present study of the rattlesnake myotoxins because of the consistent observations of delta lesions in rattlesnake venom

induced myonecrosis. It has been suggested that many snake venom myotoxins may initiate damage by differing mechanisms, but the cellular responses to such injury is limited. There may be a point at which the cells appear similar despite the instigating cause of injury.

Another group of myotoxins that has been isolated from snake venoms contains the phospholipase A_2 myotoxins. These toxins fall into two large groups: neurotoxic and non-neurotoxic PLA_2 myotoxins. The non-neurotoxic group is subdivided into those non-neurotoxic PLA_2 myotoxins that either have phospholipase enzymatic activity and those that do not show this activity. Table 3 illustrates these relationships.

Despite the variation in their neurotoxic effects these toxins cause a similar type of myonecrosis when injected into mouse muscle. The effects described by Johnson and Ownby (1993) for a myotoxin isolated from the venom of the broad-banded copperhead (*Agkistrodon contortrix laticinctus*), ACL myotoxin, serves as a good example of the myotoxic action of most of the PLA_2 myotoxins. They described three types of lesions induced by this toxin each with a characteristic light and electron microscopic appearance.

Type I lesions were characterized by swollen sarcoplasmic reticulum observed at the EM level which led to clear vacuoles in these cells visible at the LM level. The transverse tubular structures remained intact in these vacuolated cells. The second type observed, Type II, was a "mottled" appearance to the cells. These cells contained a great disorganization within the myofibrillar

Table 3: Characteristics and Sources of PLA2 toxins.

Class	Properties	Examples	Ref.
Neurotoxic PLA2 myotoxins	Basic, single chain (about 162 a.a. residues) or complexes, enzymatically active, presynaptic neurotoxins, highly lethal compounds	crotoxin: *Crotalus durissus terrificus*	Fraenkel-Conrat *et al.* (1980); Gopalakrishnakone *et al.*, (1984); Kouyoumdjian *et al.*,(1986)
		notexin: *Notechis scutatus scutatis*	Halpert and Eaker, (1975); Harris *et al.*, (1975
		taipoxin: *Oxyuarnus scutellatus*	Fohlman *et al.* (1976); Harris and Maltin, (1982)
		Mohave toxin: *Crotalus scutulatus scutulatus*	Bieber *et al.*, (1975); Cate and Bieber, (1978)
Non-neurotoxic PLA2 myotoxins	Enymatically Active Group: Basic, single chain (about 120 a.a. residues), PLA2 structure and activity. (Asp- 49 present)	myotoxins I and III: *Bothrops asper*	Gutiérrez *et al.*, (1984a); Gutiérrez *et al.*, (1984b); Lomonte and Gutiérrez, (1989
		bothropstoxin II: *Bothrops jararacussu*	Homsi-Brandeburgo *et al*, (1988);
	Enzymatically Inactive Group: Basic, single chain (about 120 a.a. residues), PLA2 structure but no detectable enzymatic activity (Lys-49 present)	myotoxin from *Bothrops nummifer*	Gutiérrez *et al.*, (1989)
		bothropstoxin I: *Bothrops jararacussu*	Homsi-Brandeburgo *et al*, (1988); Heluany *et al.*, (1992)
		ACL myotoxin: *Agkistrodon contortrix laticinctus*	Johnson and Ownby, (1993)
		Myotoxin II: *Bothrops asper*	Lomonte and Gutiérrez, (1989); Francis *et al.* (1991)
		Basic proteins I and II : *Trimeresurus flavoviridis*	Yoshizumi *et al.* (1990); Liu *et al.* (1990); Kihara *et al.* (1992)
		Ammodytoxin L: *Vipera ammodytes*	Krizaj *et al.* (1991)

network resulting in areas of expanded cytoplasm between myofibrils. Some myofibrils even appeared to be split or broken. Interestingly, the Z-disks attached to these disorganized fibrils were normal in structure. The final type of lesion noted by Johnson and Ownby (1993) induced by the ACL myotoxin, termed Type III, consisted of hypercontracted cells. An "early" phase was characterized by a shortening of the sarcomere lengths but retention of the proper orientation and architecture of the sarcomeres and myofilaments. However, as the contraction proceeded to later phases, this organization was lost, ultimately ending in hypercontraction of such extent that no sarcomeres could be distinguished in these areas. At the light microscopic level the affected cells had the appearance of being tightly clumped with myofilaments that appeared to have been pulled away from the basement membrane leaving areas of amorphous material.

Although snake venom toxins have direct, myotoxic actions, myonecrosis can occur secondarily to, or independently from the action of these toxins. Mechanisms that are known to contribute to myonecrosis apart from the action of venom components could be termed indirect myotoxic factors. Two indirect myotoxic factors are damage due to hypoxia and damage due to inflammatory responses. The specific roles that hypoxia and inflammation may play in snake bite induced myonecrosis are still not understood. However, there are several well characterized effects of each of these mechanisms which must certainly play some role in the general toxicity of snake venoms. Because these two indirect myotoxic factors may be so important in the study of the pathogenesis of

snake venom induced myonecrosis; they are briefly discussed here.

Hypoxia occurs when the oxygen supply to a cell or group of cells is decreased. This decrease in available oxygen leads to compensatory mechanisms within the cell and, if prolonged, can lead to irreversible damage and cell death. In fact, ischemia (the partial or complete interruption of blood flow to living tissue) resulting in hypoxia is considered to be possibly the single most common cause of cell injury (Slauson and Cooper, 1990). Ischemia can result from complete blockage of a blood vessel either up or down stream from the site of injury. Cessation of blood flow down-stream will lead to a passive congestion of blood vessels and stagnation of blood in an area. When this blood has given up its oxygen load and yet has no way to be replaced by fresh oxygen, the total oxygen tension in the tissue drops leading to ischemia. Likewise, when blood cannot reach an area with its load of fresh oxygen, the oxygen concentration is decreased to the area (often, when this blockage is complete, the resulting necrosis is termed an infarction).

Hemorrhagic toxins induce severe disruption of endothelial cells of blood vessels and massive local hemorrhage (Ownby, 1990). Blood flow and oxygen tension are decreased in the area. Therefore, hypoxia due to ischemic insult may play an important role in myotoxic effects of snake venom, especially in those venoms that induce massive amounts of hemorrhage (*Crotalus* and *Bothrops* species are excellent examples).

There are, however, certain hallmarks of hypoxia that allow distinction between direct effects of the venom components from an indirect hypoxic effect.

The most noteworthy of these characteristics is time. Hypoxia following an ischemic episode usually requires some time to develop in a tissue. Also important to note is the fact that in many instances of myonecrosis produced by purified myotoxins such as myotoxin a, although the myonecrosis is severe, there is little or no hemorrhage present in the tissue (Ownby, 1990; Mebs and Ownby, 1990). This suggests that the primary insult is not directly related to cessation of blood flow and resultant hypoxia but must be due instead to a direct action of the myotoxin.

The role of inflammation as a host response to injury has been studied in detail. However, the specific roles the inflammatory response and inflammatory mediators play in the host response to snake venom induced injury has only just begun to be investigated. Lomonte *et al.* (1993) used the mouse footpad model to investigate edema formation, hematological changes and cytokine release induced in mice when injected with *Bothrops asper* (Fer de lance) venom. Histological evaluation of these mice revealed the presence of a predominately polymorphonuclear cell infiltrate at six hours post-injection. At 24 and 72hr the inflammatory infiltrate increased with the appearance of mononuclear phagocytes (primarily macrophages). Considering the role of these cell types in the inflammatory process, it is not unreasonable to suggest that some necrosis of the adjacent muscle cells may be due to the action of lysosomal enzymes and possibly even the formation of oxygen radicals. Hansen and Stawaski (1994) demonstrated that neutrophils exacerbate the injury to isolated cardiac myocytes in culture after anoxia-reperfusion injury particularly through the production of

oxygen radicals, proteases and direct adhesion via CD11/18 adhesion complexes. This action of neutrophils is highly suggestive of an *in vivo* mechanism of myocyte destruction which may play a role in the myonecrosis induced by rattlesnake venoms. Neutrophils and macrophages may also be a source of damage by the mechanisms known as "frustrated phagocytosis". Frustrated phagocytosis is a potentially harmful event that occurs when phagocytes are unable to completely engulf areas of necrotic debris and therefore release their degradative enzymes into the local vicinity. This causes significant cellular damage and liquefaction of tissue.

Lomonte *et al.* (1993) observed hematological changes suggestive of inflammation such as a moderate leukocytosis and lymphopenia and a significant increase in interleukin-6 (IL-6). IL-6 is produced by many cell types including macrophages, endothelial cells, and fibroblasts and T-lymphocytes. It serves many diverse functions in the inflammatory response and in immunomodulation. IL-6 has also been shown to stimulate myoblasts to proliferate in culture (Austin and Burgess, 1991).The increased concentrations of interleukin-6 noted by Lomonte *et al.* (1993) as well as its known functions in inflammation suggest a vital role for this cytokine in the pathogenesis of myonecrosis and possibly the regenerative response that occurs after the damage has been done. The production of this and other cytokines in response to snake venom injection gives an additional level of complexity to the pathogenesis of myonecrosis induced by rattlesnake venoms.

Even though much has been done to characterize the myotoxic activity of

venoms from members of many species within both the *Crotalus* and *Agkistrodon* genera, very little has been done to isolate such activities from the venoms of members of the*Sistrurus* genus. The goal of the present work was to describe the pathogenesis of myonecrosis induced by crude venom from a subspecies of*Sistrurus miliarus* which is native to the Southern United States: *S. miliarus barbouri* (the Dusky pygmy rattlesnake). Two methods were used in this study. First, histopathologic examination was performed on muscle taken from mice injected with crude venom. This muscle was visualized by microscopy at both the light and electron microscopic levels. Three different types of tissue sections were examined (1) thick, 6 μm, paraffin sections stained with hemotoxylin and eosin (for light microscopy and evaluation of inflammatory reactions); (2) thick, 300-400nm, plastic embedded sections stained with methylene blue-Azure II (for light microscopic evaluation of myonecrosis and photography); and (3) thin, 30-40nm, sections stained with lead citrate and uranyl acetate for use in electron microscopic evaluation. The goal of the microscopic studies was to establish a progression of the lesions produced in muscle cells over several time periods (15 and 30 min, 1, 3, 6, 12, 24, 48, 72, 96 hr and 1, 2, and 4 wk) along with the progression of the inflammatory and regenerative responses of the animal. Second, knowing that the venoms of snakes within the Crotalidae family contain highly immunologically cross-reactive compounds (Ownby and Colberg, 1990; Bober *et al.*, 1988) and that many of these have been isolated (Mebs and Ownby, 1990) and used in the production of antibodies specific for these toxins (Ownby *et al.*, 1979); it was hypothesised

that these antibodies raised against toxins from venoms of snakes in the other crotalid genera may cross-react with immunologically similar toxins present within the venoms of members of the *Sistrurus* genus. If present, these immunologically similar toxins may be important in the lesion observed in muscle cells. Since much is known about the structure and biologic activity of these toxins, reasonable theories can be proposed concerning the possible mechanisms that leading to the microscopic lesions produced by crude venoms of the *Sistrurus* genus.

CHAPTER 2

Materials and Methods

Venom, animals, injections

Crude *Sistrurus miliarus barbouri* venom was purchased from Miami Serpentarium (Salt Lake City, Utah, U.S.A.) in lyophilized form and kept at 0°C until used. Two specimens of *Sistrurus miliarus streckeri* maintained at the Oklahoma State University Serpentarium were also extracted and the crude venom lyophilized and stored at 0°C. All venom samples were reconstituted into physiological saline (0.85% NaCl) immediately prior to injection.

Adult female white mice (CD-1, Charles River) were purchased and upon arrival were allowed to acclimate for two or three days. Mice were weighed before injection and appropriate volumes of venom solution were injected at a final dose of 3.5µg venom/g body weight (~0.05µL total volume injected). The dosage of 3.5µg venom/g body weight was chosen because it is substantially below the reported LD_{50} (6.84 µg/g i.p. in mice; Tu, 1982) of the venom but still produced significant myonecrosis. All injections were made in the caudomedial aspect of the right thigh. Mice were then killed at varying time periods and the muscle tissue removed.

Muscle was taken from each mouse at one of 13 time periods (15 and 30

injected for each period (26 experimental animals) along with two control animals for each tissue processing group. Tissue was then processed for light and electron microscopy.

Microscopy

Plastic embedded sections

Skeletal muscle tissue from each mouse was fixed immediately in 2% EM grade gluteraldehyde in 0.27 M cacodylate buffer (pH 7.2), fixed again with OsO4 , dehydrated stepwise in ethanol (from 10% to absolute), stained *en bloc* with uranyl acetate overnight and embedded in plastic resin (Polybed 810). After curing for 3 days, thick sections (~400nm) were cut using a Sorvall MT-5000 ultramicrotome and stained with methylene blue for light microscopic evaluation. Those sections used for EM evaluation were thin sectioned (~60 nm) using a Sorvall MT-6000 ultramicrotome, stained with lead citrate and uranyl acetate and evaluated by electron microscopy using a JEOL 100 CX scanning transmission electron microscope.

Paraffin Sections

Paraffin sections of skeletal muscle from mice treated with 3.5µg/g crude *Sistrurus miliarus barbouri* or *S. miliarus streckeri* venom at 30 min, 3, 6, 12, 24, 48, 72 and 96 hr were made by the Histopathology Department of the Oklahoma Animal Disease Diagnostic Laboratory (OADDL), Oklahoma State University

College of Veterinary Medicine, Stillwater, OK, U.S.A. and stained with hematoxylin and eosin for light microscopic evaluation for correlation with methylene blue stained sections as well as interpretation of host responses such as inflammation and immune responses.

Immunoblotting

Immunoblotting techniques were used for these studies. These methods involved subjecting various venoms from specimens of several genera of the Crotalidae family to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferring the protein to nitrocellulose membranes by electroblotting and incubating the membranes with specific antibodies against purified toxic components from other venoms to determine if the specific antibodies would cross-react to any of the components of *Sistrurus miliarus barbouri* venom.

Samples of venom from four genera of the Crotalidae family were used. These were chosen based upon known characteristics of the venoms especially the presence of myotoxic components which have been isolated, purified and to which specific antisera has been raised. The venoms were then used to assay the immunological cross-reactivity of *Sistrurus miliarus barbouri* to these isolated components.

The venoms used in this study were (1) *Agkistrodon contortrix laticinctus* (Broad-banded Copperhead) due to the presence of the ACL myotoxin described by Johnson and Ownby (1993) as a PLA_2 myotoxin; (2) *Bothrops jararacussu* due to the presence of bothropstoxin I, a PLA_2 myotoxin lacking enzymatic

activity (Homsi-Brandeburgo *et al.*, 1988;Cintra *et al.* , 1993); (3) *Sistrurus miliarus barbouri* (Dusky pigmy rattlesnake); (4) *Sistrurus miliarus streckeri* (Western Pygmy Rattlesnake) ; (5)*Crotalus horridus horridus* (Timber Rattlesnake): (6) *Crotalus viridis viridis* (Prarie Rattlesnake) from which myotoxin *a* was isolated (Ownby *et al.*, 1976; Cameron and Tu, 1977); and (7) *Crotalus durissus terrificus* (South American Rattlesnake) due to the presence of the enzymatically active PLA_2 myotoxin, crotoxin (Gopalakrishnakone *et al.*, 1984).

Three purified toxins: myotoxin *a* (isolated according to Ownby and Colberg, 1987), crotoxin B (gift of Dr. Cassian Bon, *Institute Pasteur*, Paris France) and ACL myotoxin (from Johnson and Ownby, 1993) served as positive controls in immunoblotting. Specific antisera to each of the above three myotoxins were obtained as follows: anti-myotoxin *a* serum courtesy of Dr. Charlotte Ownby, anti-crotoxin B courtesy of Dr. Cassian Bon (*Institue Pasteur* , Paris, France) and anti-ACL myotoxin serum from Li and Ownby (1994) polyclonal serum. These sera were used as immunologic probes in subsequent Western blots.

Electrophoresis for Western blots was performed on a Pharmacia Phastsystem using Pharmacia PhastGels with an 8-25 concentration gradient. The venom samples as well as purified myotoxins were dissolved in a sample buffer containing 0.01 M Tris-HCl (pH 8.6) and 5% sodium dodecyl sulfate for non-reducing and 5% 2-mercaptoethanol was added to the buffer for the

was performed for 80 volt-hr following the recommended Phastsystem protocol. Gels were stained with Coomassie Blue or used for Immunoblotting.

Gels from SDS-PAGE were transferred to nitrocellulose membranes using the PhastSystem electroblotting technique following the Protein Transfer Protocol. After protein transfer, the membrane was placed in a blocking buffer containing 0.01 M Tris Buffered Saline with 0.01% Tween-20 (TTBS) and 3% gelatin and incubated at room temperature for 3 hr to block non-specific binding sites. After washing with TTBS, the membrane was placed in a vessel containing hyperimmune serum from rabbits immunized with purified myotoxin a, crotoxin or ACL myotoxin buffered in TTBS with 1% gelatin. After incubation for 4 hr at room temperature, the membrane was again washed (twice with TTBS and once with TBS to remove tween).

The bound antibodies were visualized using the Protein A Gold Immuno-Blot Assay Kit (Bio-Rad Labs., Richmond, CA, U.S.A.) and enhanced with Gold Enhancement Kit also from Bio-Rad following recommended protocols. Membranes were then dried and photographed.

CHAPTER 3

Results

Crude *Sistrurus miliarus barbouri* venom induced necrosis in mouse skeletal muscle after i. m. injection. The lesions induced could be classified into three general categories based on the presence or absence of inflammatory infiltrate in the tissue: "pre-inflammatory changes", "inflammatory changes" and "post-inflammatory" changes. "Pre-inflammatory" is defined as those morphological changes that occur in the tissue before the mobilization and migration of inflammatory cells (predominately neutrophils, polymorphonuclear cells or PMNs). "Inflammatory" changes are defined as those changes that occur in the presence of an acute, obvious inflammatory infiltrate in the tissue, whereas, "post-inflammatory" changes occur after the cessation of the acute inflammatory response and are generally limited to the regenerative responses.

The major changes that occurred in the pre-inflammatory period (15 min to 3 h.) were massive hemorrhage and edema and severe local myonecrosis. Hemorrhage was indicated by the presence of a large number of extravasated erythrocytes in the interstitial spaces. The presence of extremely congested blood vessels was also noted. Generally, hemorrhage was present from 15 min-6 hr and was mostly resolved by 12-24 hr. However, some capillaries remained intact. Interstitial edema was indicated by a great expansion of the intercellular spaces and connective tissue. Much flocculent material (presumably fibrin and

coagulated plasma) was observed in the connective tissue spaces (Figure 1). Myonecrosis was indicated by the appearence of muscle cells exhibiting various pathological states. Four different pathological states mark the pre-inflammatory period and have been previously described (Ownby and Colberg, 1988; Johnson and Ownby, 1993). This discussion will use these same designations to avoid confusion. The four types of damaged cells that are present in the pre-inflammatory period are (1) cells with delta lesions (triangular shaped areas of clearing in the muscle cells oriented with the point of the triangle toward the center of the cell), (2) cells with densely clumped myofilaments alternating with clear areas within the cell, (3) cells with hypercontracted myofilaments and (4) cells that have disorganized or "broken looking" myofibrils. (Figure 2)

The inflammatory period (6 to 96 hr) was marked by an extensive cellular infiltrate (especially at 6 and 12 hr post-injection) which consisted mostly of polymorphonuclear (PMN) leukocytes (Figure 3). Aside from the extensive myonecrosis which is discussed below in detail; the presence of neutrophils was the most obvious change occurring in the tissue during the inflammatory period. All four types of muscle cell lesions present in the pre-inflammatory period were still present in the cells and were accompanied by the presence of frankly necrotic cells recognizable by their amorphous disorganized appearance and pyknotic nuclei. In the earlier part of the period, 6- 12 hr, the necrotic cells were often surrounded and infiltrated by neutrophils (Figure 3). However, in the later part of the inflammatory period (24- 96 hr) macrophages became the primary

Figure 1: Light micrograph of mouse skeletal muscle 30 min after i.m. injection of crude *Sistrurus miliarus barbouri* venom. Note extravasated erythrocytes (E), damaged muscle cells (dM) and flocculent material in extracellular spaces (F). (Paraffin section, H&E stain, 45X)

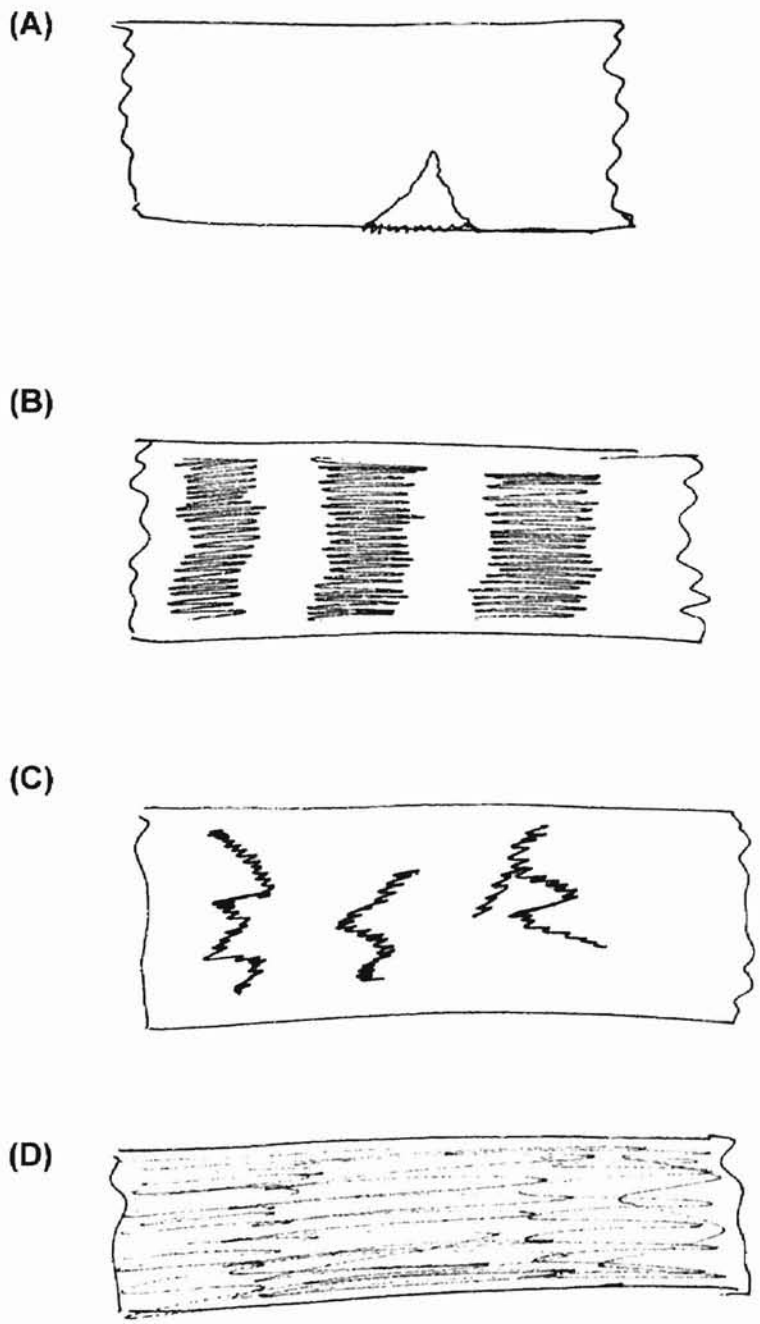


Figure 2: Diagrammatic representation of the four histological types of muscle cell damage seen during the inflammatory period. (A) Cells with delta lesions: (B) cells with densly clumped myofibrils; (C) cells with hypercontracted myofilaments and (D) cells with amorphous necrotic material.

inflammatory cell type present while fibrosis became more evident (Figure 4).

The later part of the inflammatory phase (48-96 hr) was characterized by regeneration of damaged cells. Figure 5 illustrates several regenerating cells (notable by their smaller size and central nuclei) Several of these cells exhibit "nuclear streaming", a term used to describe the lining up of several nuclei in a central location within the regenerating cel, which is another sign of rapidly growing muscle cells.

The post-inflammatory phase (1-4 wk) was dominated almost entirely by regenerating cells although a few remnants of necrotic cells could be seen . None of the previously existing types of lesions were noted in the post-inflammatory phase although there were probably areas of the tissue that were still progressing through the stages of necrosis. As the tissue progressed through the post-inflammatory stage, the regenerating cells grew in size and began to appear closer to normal. At four weeks after the insult, most of the tissue had been restored to normal (Figure 6).

Immunoblotting

Immunoblotting using antisera raised against purified myotoxin *a*, ACL myotoxin and crotoxin B yielded interesting results. These antibodies reacted strongly with both of two positive controls (the purified toxin and the homologous crude venom) in each of the three experiments. Specifically, when the seven crude venoms (listed in Materials and Methods) were exposed to antibodies

Figure 3: Light micrograph of mouse muscle 3 hr after i.m. injection of crude *Sistrurus miliarus barbouri* venom. Note polymorphonuclear cells (PMN) and damaged muscle cells (dM). (Paraffin section, H&E stain, 40X)

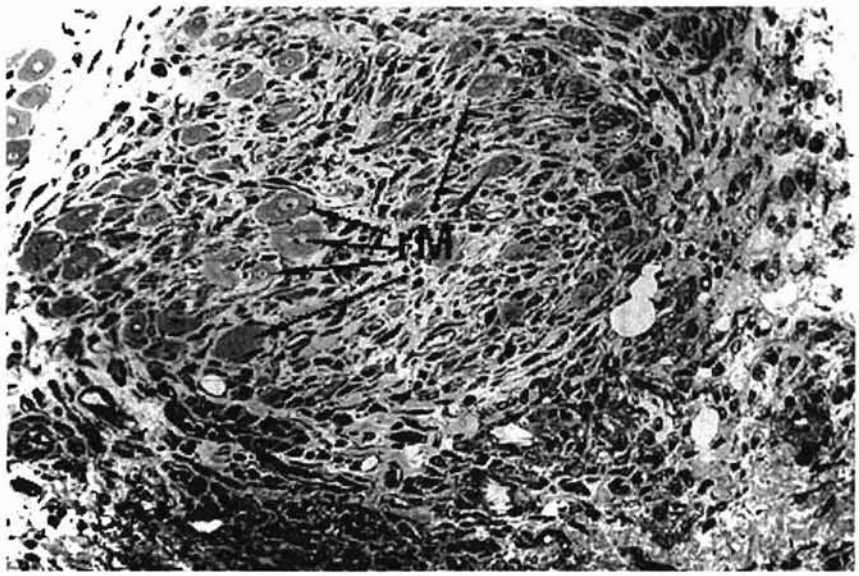


Figure 4: Light micrograph of mouse muscle 1 wk after injection of crude *Sistrurus miliarus barbouri* venom. Note regenerating muscle cells (rM) surrounded by an area of fibrosis. (Plastic section, Mallory's staining, 40X)

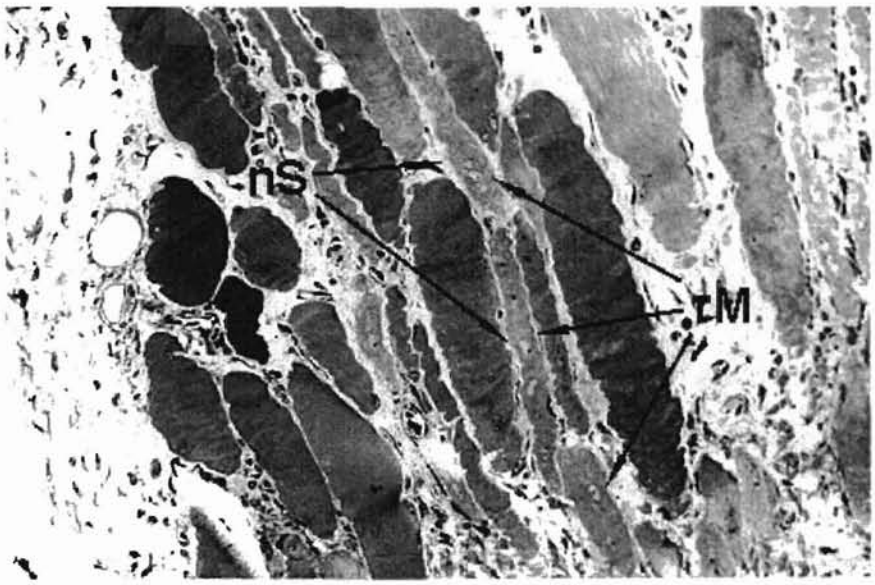


Figure 5: Light micrograph of mouse muscle 2 wk after i.m. injection of crude *Sistrurus miliarus barbouri* venom. Note regenerating muscle cells (rM) with central nuclei, nuclear streaming (nS) and necrotic intracellular debris. (Plastic section, Mallory's stain, 40X)

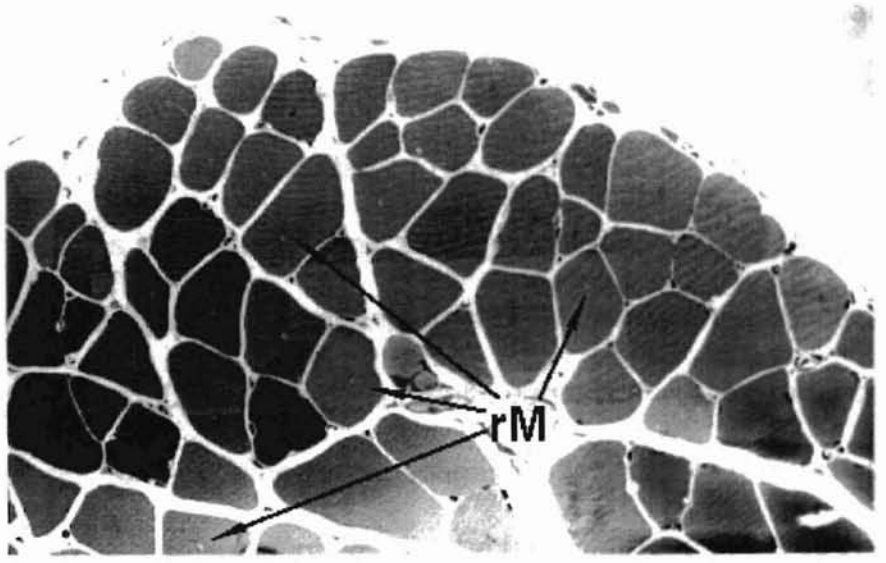


Figure 6: Light micrograph of mouse muscle 4 wk after i.m. injection of crude *Sistrurus miliarus barbouri* venom. Note regenerating muscle cells of normal size with central nuclei (rM) and normal tissue architecture. (Plastic section, Mallory's stain, 40 X)

raised to purified myotoxin *a*, only the pure myotoxin *a* control sample and homologous crude venom (*C. viridis viridis*) reacted positively under both reducing and non-reducing conditions (Table 4, Figure 7). This result suggests that of the venoms incubated against anti-myotoxin *a* antibodies only the myotoxin *a* in the positive control and present in the crude venom cross-reacts with the antibodies. Conversely, this result suggests that *S. miliarus barbouri* venom does not contain components antigenically similar to myotoxin *a* . This conclusion can also be substantiated by the fact that none of the specific lesions described in the pathogenesis of myonecrosis induced by myotoxin *a* (dilation of sarcoplasmic reticula and perinuclear spaces) were noted in this study with venom from *Sistrurus miliarus barbouri*. The fact that there was cross-reactivity under both reducing and non-reducing conditions suggests that the antigenic determinants on the myotoxin *a* molecule are linear and not conformational. This may become important in elucidating the mechanism of action of the toxin in the future.

When the venoms were exposed to antibodies raised against purified ACL myotoxin, the only positive reactions were seen with the homologous crude venom (*A. c. laticinctus*) under non-reducing conditions, but a weakly positive result was observed for *S. m. barbouri* crude venom and for *C. h. horridus*. venom under reducing conditions (Table 5, Figure 8). These results are rather confusing, but may be suggestive of an epitope buried within the three dimensional structure of a protein within *S. miliarus barbouri* venom and *C. h. horridus* venom to which the anti-ACL myotoxin antibodies cross-react. There is

evidence from the histologic studies that *S. miliarus barbouri* venom is capable of producing myonecrosis that is similar in appearence to that produced by *A. c. laticinctus* venom, yet this has not been definitively established.

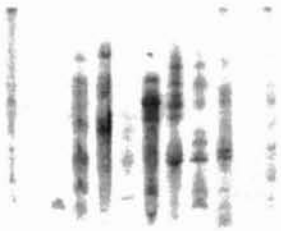
When venoms were reacted with antiserum to crotoxin B, four venoms reacted positively, including *S. miliarus barbouri* venom suggesting that *S. miliarus barbouri* venom contains a protein antigenically similar to crotoxin B. The non-homologous venoms only reacted under non-reducing conditions suggesting that the reacting epitope is probably conformational and not linear.

Table 4: Summary of Reactions Obtained on Immunoblots Shown in Figure 7 Using Antiserum to myotoxin *a*.

Venom or Toxin	Non-reducing conditions	Reducing Conditions
Myotoxin-a	+	+
A. contortrix laticinctus	-	-
B. jararacassu	-	-
S. miliarus barbouri	-	-
S. miliarus streckeri	-	-
C. horridus horridus	-	-
C. viridis viridis	+	+
C. durissus terrificus	-	-

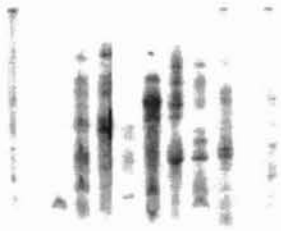
+ = Bands present

- = No bands present



SDS-PAGE nonreducing conditions

Immunoblot against anti-MyoA



SDS-PAGE reducing conditons

Immunoblot against anti-MyoA

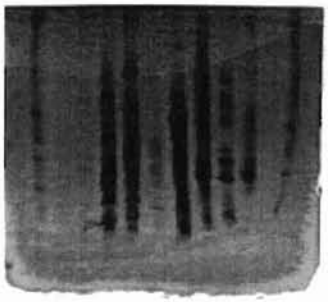
Figure 7: SDS-PAGE Showing Electrophoretic Profiles Obtained with Venoms and Immunoblots Showing the Reaction Obtained with Anti-myotoxin *a* serum.

Table 5: Summary of Reaction Obtained on Immunoblots shown in Figure 8 Using Antiserum to ACL Myotoxin.

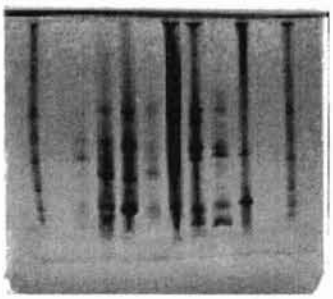
Venom or Toxin	Non-reducing conditions	Reducing Conditions
A. c. l. myotoxin	+	+
A. contortrix laticinctus	+	+
B. jararacassu	-	-
S. miliarus barbouri	-	-
S. miliarus streckeri	-	-
C. horridus horridus	-	-
C. viridis viridis	-	-
C. durissus terrificus	-	-

+ = Bands present

- = No bands present



SDS-PAGE nonreducing conditions Immunoblot against anti-ACL myotoxin



SDS-PAGE reducing conditions Immunoblot against anti-ACLmyotoxin

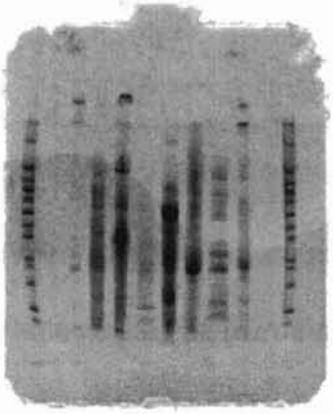
Figure 8: SDS-PAGE Showing Electrophoretic Profiles Obtained with Venoms and Immunoblots Showing the Reaction Obtained with Antiserum to ACL myotoxin.

Table 6: Summary of Reactions Obtained on Immunoblots Shown in Figure 9 Using Antiserum to Crotoxin B.

Venom or Toxin	Non-reducing conditions	Reducing Conditions
crotoxin B	+	+
A. contortrix laticinctus	+	-
B. jararacassu	+	-
S. miliarus barbouri	+	-
S. miliarus streckeri	-	-
C. horridus horridus	-	-
C. viridis viridis	-	-
C. durissus terrificus	+	+

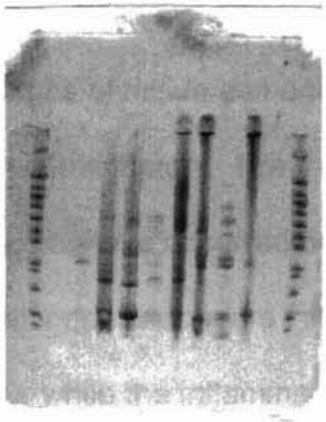
+ = Bands present

- = No Bands present



SDS-PAGE nonreducing conditions

Immunoblot against Crotoxin-b



SDS-PAGE reducing conditions

Immunoblot against Crotoxin-b

Figure 9: SDS-PAGE Showing Electrophoretic Profiles Obtained with Venoms and Immunoblots Showing the Reation Obtained with Antiserum to Crotoxin B.

CHAPTER 4

Discussion

When injected intramuscularly into mice, crude venom from the Dusky pygmy rattlesnake (*Sistrurus miliarus barbouri*) incited a complex series of pathological changes. These changes included edema, hemorrhage , myonecrosis and inflammation. Inflammatory responses served as the basis for description of general stages of tissue damage induced by the crude venom. Three stages of tissue damage were observed: pre-inflammatory, inflammatory and post-inflammatory. Pre-inflammatory changes are those pathological changes that have occurred before the histologic evidence of infiltration of inflammatory cells appears in the tissue. Inflammatory changes refer to those that occur while the inflammatory infiltrate is present in the tissue and presumably actively participating in the overall progression of tissue damage. Post-inflammatory changes occur after the infiltrate is no longer predominant in the tissue.

Lomonte *et al.* (1993) used the mouse footpad model to investigate the inflammatory responses (specifically the formation of edema, infiltration of inflammatory cells and release of cytokines) in mice when injected with *Bothrops asper* (Fer de lance) venom. They observed local edema and inflammatory cell infiltration with the presence of a predominately polymorphonuclear cell infiltrate at six hr post-injection in these mice. This compares favorably with the

observations made in the present study with *S. m. barbouri* venom. Lomonte *et al.* (1993) also observed that at 24 and 72hr the inflammatory infiltrate increased with the majority of infiltrating cells consisting of mononuclear phagocytes, and that several important cytokines were released during the inflammatory response elicited by venom from *Bothrops atrox*. These types of changes were observed in the present work and lead to some interesting suggestions as described below concerning the mechanisms surrounding the development of edema, hemorrhage, myonecrosis and inflammation resulting from venomous snake bite.

Edema, specifically interstitial or intercellular edema, is a common clinical observation in cases of crotalid snake bite (Gomez and Dart, 1995) and was observed both grossly and histologically in this study. According to Ownby (1982), most pathologists classify the majority of edema produced by rattlesnake envenomations as a serous inflammatory exudate or inflammatory edema. Lomonte *et al.* (1993) studied the host inflammatory response and edema producing effects of crude fer-de-lance (*Bothrops asper*) venom and found that two types of edema were produced. At low doses (~1 µg) a "rapid, but transient" edema was observed with no signs of hemorrhage and a very mild inflammatory infiltrate in the mouse footpad model. However, at higher doses (~50 µg) the edema observed had just as rapid an onset, but was much more severe and was accompanied by hemorrhage, a massive inflammatory infiltrate and myonecrosis. This observation led Lomonte to suggest that the response to the low dose of venom was more likely a direct effect of venom components whereas

the response to the higher dose was likely a combination of the venom and inflammatory processes. This dose dependent response of the tissue to differing concentrations of venom led Lomonte *et al.*, (1993) to propose that the degree and duration of edema present in the tissue may be a useful gauge for estimation of the severity of a human envenomation.

The dose of venom from *S. m. barbouri* used in this study was 3.5 µg/g body weight which is half of the reported LD_{50} for *S. m. barbouri* venom (Tu, 1982). At this dosage, edema was very pronounced by 15 min and did not appear to be associated with an inflammatory cell infiltrate. However, by 3 hr (and certainly very pronounced at 6 hr) the edema persisted and was accompanied by a large number of inflammatory cells. These results were very similar to those presented above (Lomonte *et al.*, 1993).

Another characteristic clinical finding associated with bites from crotalid snakes is hemorrhage. Also a characteristic local effect induced by *Sistrurus miliarus barbouri* venom, hemorrhage was present in the tissue both grossly and upon histologic evaluation. Hemorrhage observed after injection of crude *Sistrurus miliarus barbouri* venom was characterized by extravasation of erythrocytes into the interstitial spaces. In many areas of the tissue, there was evidence of flocculent material that could be fibrin or coagulated plasma. It was, however not organized and appeared to have leaked out of the vessels. In none of the sections were any platelet plugs observed at the light microscopic level. In association with areas of hemorrhage there were vessels that appeared to be intact, but were very congested suggesting either increased blood flow to the

area or a stagnation of flow and passive congestion of the vessels. Considering the great extent of the hemorrhage observed, the latter possibility seems most likely. The venom from *Sistrurus miliarus barbouri* clearly resembles other venoms from snakes in the Crotalinae subfamily of the Viperidae in its ability to induce massive hemorrhage.

Many hemorrhagic toxins have been isolated from crotalid venoms (Bjarnason and Fox, 1994), however; none have been isolated from the venom of *Sistrurus miliarus barbouri* . However, a specific protein which may potentiate the development of hemorrhage has been isolated from this venom. It has been named "barbourin" , and it is a unique member of the "disintegrin" family of proteins that have been isolated from viperid venoms (Scarborough *et al.,* 1991; Scarborough *et al.,* 1993). Several of these peptides exist in the venoms of the snakes that are members of the Crotalinae subfamily and they are believed to be autoproteolytic modifications of hemorrhage producing metallo-proteinases present in the crude venom (Takeya *et al.,* 1993; Bjarnason and Fox, 1995). They are of importance due to their specific action against adhesion or docking proteins (integrins) on platelets and their modulation of the activity of fibrinogen and von Willebrand factor (Scarborough *et al.*, 1993). They are also known to inhibit platelet aggregation and therefore, may contribute to the hemorrhagic effect of these venoms. In fact, some scientists believe that it is the action of these proteins on platelets that may account for the entire hemorrhagic effect of *Bothrops jararaca* venom and may be the mechanism of hemorrhage induced by other crotalid venoms (Kamiguti *et al.*, 1991).

All of the disintegrin molecules from Viperid venoms (except barbourin) that have been studied thus far have a similar amino acid sequence, RGD (Arg-Gly-Asp), that appears to be very important in the activity. These peptides show a specificity for integrin molecules including integrins such as the GPIIb-IIIa glycoprotein (present on platelets and megakaryocytes) and regions on fibrinogen and von Willebrand factor (Scarborough *et al.*, 1993). Thus, they may be important in preventing coagulation. Barbourin is the only disintegrin peptide isolated thus far that has a KGD (Lys-Gly-Asp) sequence at this site. This slight modification appears to be all that is necessary to impart upon this peptide exquisite specificity for GPIIb-IIIa integrins. It could be suggested then, that the presence of this disintegrin in *Sistrurus miliarus barbouri* venoms may be responsible for some of the hemorrhage observed by interfering with the adherence of platelets to the vessel walls and to each other. Although the presence of barbourin in the venom of *Sistrurus miliarus barbouri* could very well explain some of the hemorrhagic activity, more detailed and specific research must be performed in order to estimate the importance of this peptide in causing the severe hemorrhage observed *in vivo*.

Other than barbourin, no specific toxins have been isolated from the venom of *Sistrurus miliarus barbouri*. However, based upon the myonecrosis observed and the results of immunoblotting against antibodies raised to myotoxins from other venoms, there is evidence that *S. m. barbouri* crude venom contains specific myotoxins. The myotoxins present in *S. m. barbouri* crude venom may very well be of the phospholipase A_2 (PLA_2) type. The crude

venom produced lesions identical to those observed for other PLA_2 myotoxins like the ACL myotoxin (Johnson and Ownby, 1993), *B. asper* myotoxin (Gutiérrez *et al.,* 1990), bothropstoxin-II (Gutiérrez *et al.*, 1991), and others. The effects of these toxins are characterized by muscle cells with densely clumped and hypercontracted myofilaments, delta lesions and contraction banding when observed at the light microscopic level. The lesions induced by crude *S. miliarus barbouri* venom follow a pattern very similar to that induced by other crotalid venoms. The myonecrosis observed was part of a continuum of damage to individual cells which were challenged by toxins within the crude venom. Ownby and Colberg (1988) proposed a time-table or sequence of events concerning the pathogenesis induced by *Naja naja naja* (Indian Cobra), *Crotalus viridis viridis* (Prairie rattlesnake) and *C. atrox* (Western Diamondback rattlesnake) crude venoms in mouse skeletal muscle. They suggested that five different morphological appearances were present in the "early period" (0.25 h to 3 h) then passed through an "intermediate" period (3-6 h) where two other types of lesions were noted. The cells then progressed to a "late phase" between 48-96h in which all the damaged cells had reached a common stage of degeneration noted by an amorphous appearance associated with the presence of phagocytes. The "final phase" (1-4 wk) was marked by regeneration and normal healing of the tissue. The classifications used here follow and expand on this description.

In the present study of *S. m. barbouri* venom, several distinct lesions in skeletal muscle cells were noted. These have been classified as 1) delta

lesions, 2) densely clumped myofilaments, 3) hypercontracted myofilaments, 4) "broken myofilaments". In previous studies, these have been characterized as different types of lesions induced by the toxins. The present study reveals that these are progressive stages of damage occurring within a single cell. These results agree with those of Johnson and Ownby (1993) who also suggested that information obtained from the cross-sectional appearance of the necrotic cells was limited. When longitudinal sections of these cells were observed, different types of damage could be seen along the length of the cells. In the present study care was taken to provide longitudinal as well as cross sections of the tissue from each time period. Additionally, separate sections were made of paraffin embedded tissue that were much larger and thicker than the previously mentioned plastic sections thereby allowing a much greater appreciation for the extent and type of damage than the thinner, more restricted plastic sections afford. With the differential nature of hematoxylin and eosin staining, better estimations of the type and extent of inflammatory responses could be made. When these two methods were employed simultaneously, a very clear picture of myonecrosis induced by crude venom was obtained.

In an intravital study (Lomonte *et al.*, 1994), the first detectable lesions to develop after venom injection into isolated mouse cremaster muscle preparations were delta lesions which are triangular-shaped areas of clearing within the damaged cells in which the point of the triangle faces toward the center of the cell and the base of the triangle lays along the plasma membrane . These types of lesions have also been described in biopsy specimens from

humans with Duchenne muscular dystrophy (Mokri and Engel, 1975). This observation is consistent with the present work in which delta lesions were the first noticeable changes seen in the cells.

It has been shown that the PLA_2 myotoxins induce skeletal muscle damage by first affecting the integrity of the cell membrane (Gutiérrez *et al.*, 1984a, b; 1986, Lomonte and Gutiérrez , 1989; Johnson and Ownby, 1993). The damage could be induced by several mechanisms such as a detergent-like dissolution of the membrane, the formation of membrane pores that disrupt the osmotic balance of the cells or possibly enzymatic degradation of the membrane phospholipids. Whatever the mechanism, it is clear that the damage is severe enough to allow the efflux of creatine kinase and creatinine into the plasma as well as allow a great influx in Ca^{2+} (Gutiérrez *et al.*, 1984a) and other ions. The myotoxins isolated from the crude venom of *Bothrops asper* include both enzymatically active and enzymatically inactive PLA_2 types of toxins (Gutiérrez and Lomonte, 1989; 1995). Although the action of a PLA_2 directly upon the membrane seems a reasonable and understandable mechanism for the initiation of this type of damage, some myotoxins (such as the ACL myotoxin) cause very similar types of lesions as described for the PLA_2 type of toxins but they have no detectable enzymatic activity. In fact, the majority of toxins in crude *Bothrops asper* venom are of the nonenzymatic PLA_2 type and yet, still account for almost 75% of the total myotoxicity of the venom (Lomonte *et al.*, 1985; Lomonte *et al.*, 1987). It is obvious from this that all of the myotoxicity of these proteins is not due only to enzymatic activity. These results suggest that there may be

other signaling mechanisms involved in the development of the lesions induced in skeletal muscle cells by these toxins.

Two mechanisms could be proposed as possible mechanisms of action of these toxins. Both of these assume that an increase in intracellular Ca^{2+} concentration is required for the contraction of myofilaments within the myocyte. The first of these two proposals could be called the "diffusion" theory. It suggests that the major driving force of a rise in intracellular Ca^{2+} is an influx of Ca^{2+} down it's concentration gradient into the cell after disruption of its permeability barrier. Johnson and Ownby (1993) suggested that this increase in intracellular Ca^{2+} ions causes the contraction of actin-myosin complexes within the skeletal muscle cells and is responsible for the initial contracture of myofilaments observed due to the PLA_2 toxin, ACL myotoxin. As the intracellular Ca^{2+} concentration increases, the myofibrillar elements continue to contract and shortly assume this hypercontracted state. This type of pathogenesis has also been proposed by Harris and Cullen (1990). However, to test the validity of this hypothesis, Johnson and Ownby (1994) incubated muscle preparations in differing concentrations of ions after inducing damage to the membranes and found that even very high Ca^{2+} concentrations (up to 200mM) did not produce the hypercontracted myofilaments that isolated ACL myotoxin produced. The fact that an experimentally induced "hole" in the membrane which is certainly nonselective to ions and a great increase of extracellular Ca^{2+} (which would be expected to force Ca^{2+} into the cell and increase the intracellular Ca^{2+} concentration) did not produce the hypercontraction of

myofilaments is highly suggestive that other factors must be considered.

Because the physiologic method for increase in intracellular Ca^{2+} in skeletal muscles leading to contraction of myofilaments is dependent upon membrane depolarization and a subsequent increase in intracellular Ca^{2+}, another proposal which could be termed the "depolarization" theory may be suggested. Normally, the activation of cholinergic nicotinic receptors at the motor end plate by acetylcholine is coupled by second messenger systems to both membrane Ca^{2+} channels and intracellular Ca^{2+} channels which are located on intracellular membranes like the sarcoplasmic reticulum. Activation of these channels leads to a depolarization which is propagated along the membrane via Na^+/K^+ co-port proteins. The method of propagation is the influx of Na^+ down its gradient and the efflux of K^+ down its gradient out of the cell. Johnson and Ownby (1994) observed that isolated muscle preparations that had been experimentally damaged and bathed in a solution containing high concentrations of NaCl produced hypercontracted myofilaments very similar to that induced by purified ACL myotoxin. This led Johnson and Ownby (1994) to propose that the effect of the ACL myotoxin may be to increase the Na^+ concentration intracellularly to such an extent that the muscle cells are depolarized and prevented from repolarization. Such sustained depolarization may travel down the T-tubular system, which is the normal route of conduction within the skeletal muscle cell, and may induce release of Ca^{2+} from the terminal cisternae of the sarcoplasmic reticulum. Thus the intracellular Ca^{2+} concentration may rise to such an extent as to produce the hypercontracted

myofilaments observed (Johnson and Ownby, 1994).

Both the diffusion theory and the depolarization theory could theoretically lead to an increase in intracellular Ca^{2+} and could possibly cause the formation of hypercontracted myofilaments. These mechanisms have yet to be definitively proven, but each has experimental merit and physiological precedent. Therefore either one or both may be operative at the time of muscle damage by toxins similar to the ACL myotoxin. Damage to cells induced by *Sistrurus miliarus barbouri* venom is very similar to that induced by the ACL myotoxin as well as other crotalid toxins of the PLA_2 class such as crotoxin B. Therefore, it seems reasonable to suggest that perhaps mechanisms like these previously mentioned may be occurring in the tissue and may be the cause of the myonecrosis observed in this study.

The damage induced by *S. m. barbouri* venom to a muscle cell appears to be "propagated" intracellularly. Whether by the diffusion of ions across the membrane and down the cell or by the depolarization of the membrane along the length of the cell (or certainly perhaps neither of these mechanisms), it seems clear that the damage is somehow transmitted throughout a cell. However, the possibility exists that the presence of several different types of lesions within one cell may simply be the result of different areas of the cell responding independently at different times to the action of toxins within the venom. Both the diffusion theory and the depolarization theory would predict, nonetheless, that the cause of damage is actively transmitted (whether by diffusion or depolarization) throughout the cell. Certainly, delta lesions are the first

indications of damage, and the damage spreads in both directions from this focal point. In longitudinal sections many single cells can be observed throughout their entire length (especially in thick paraffin sections). The occurrence of damage within single cells can be easily observed from these sections. Delta lesions appear to be focal areas of damage along the length of the cell. It seems that the damage done to a single cell at one end of that cell has been propagated to the other end leaving behind a trail of damage in a sequential pattern. For example, in a group of muscle cells sectioned longitudinally, one or two may be damaged at one end. When the full length of the section is viewed, only those two cells show any sign of damage throughout the entire length of the tissue. If the damage to muscle cells is propagated as Johnson and Ownby (1994) suggested by the sustained depolarization of the membrane (depolarization theory), one would expect to see damage occurring along the entire length of the cell, radiating out from a focus of damage to the membrane. If, as could also be the case, the damage is due to the influx of Ca^{2+} (diffusion theory) which diffuses down the length of the cell initiating the hypercontraction of myofilaments along the way, one would expect to see the effect of this diffusion along the length of the cell provided the concentration of intracellular Ca^{2+} was great enough to overcome the cellular sequestration mechanisms. In either case, it appears obvious from histologic evaluation that the damage to a cell is confined to within that cell.

The pathogenesis of myonecrosis induced by *Sistrurus miliarus barbouri* venom may indeed involve one or both of these aforementioned mechanisms as

well, and it may also include contributions from the inflammatory response as well. Research into the effects of inflammation upon necrosis induced by snake venom suggests that inflammatory processes may play a much greater role in the overall development of necrosis than previously thought. The amount of neutrophilic infiltrate into the tissue observed here suggests that neutrophils may be playing a vital role in the development of necrosis especially in the inflammatory period (6-96 hr). This idea certainly has pathological precedence as exemplified in the phenomenon of "frustrated phagocytosis". "Frustrated phagocytosis" occurs when a phagocytic cell, usually a macrophage or neutrophil, is stimulated to release it's enzymes into the surrounding tissue rather than direct the release at a specific cell. This uncoordinated release of very destructive proteolytic enzymes can cause significant damage to other cells within the area and may contribute to myonecrosis. Gutiérrez *et al.* (1990) stated that the action of neutrophil-derived proteases upon the myofibrillar elements of damaged muscle tissue (treated with isolated myotoxin from *B. asper* venom) especially at 24-72h is most likely due to the dual action of macrophages and neutrophils. This observation agrees quite well with the present findings.

In summary, there are different possible mechanisms that may be operating in the pathogenesis of myonecrosis induced by *Sistrurus miliarus barbouri* venom. The progression of damage seems to be from delta lesion to densely clumped myofilaments to hypercontracted myofilaments to disorganized myofibrils to an amorphous mass of necrotic debris. This progression certainly resembles that caused by toxins that have been studied from venoms of

members of the other genera in the subfamily Crotalinae (specifically, the genera *Crotalus, Bothrops* and *Agkistrodon*).

Myonecrosis induced by crude snake venoms may be caused by two major mechanisms 1) direct action on the cell, likely through membrane damage and 2) the effects of ischemia produced by the destruction of microvascular elements in the tissue by the venom (Gutiérrez *et al.*, 1984, Gutiérrez and Lomonte, 1989). Although very similar to the necrosis induced by crotalid venoms, the necrosis caused by *S. m. barbouri* venom also exhibits some differences. Chiefly among these differences is the apparent lack of small, basic myotoxins (like crotamine and myotoxin *a*) and the presence of the unique disintegrin, barbourin.

The results of immunoblotting performed in this study suggests the presence of PLA_2 like myotoxins in *S. m. barbouri* venom based on the reactivity of specific antisera against two PLA_2 myotoxins (ACL-myotoxin and crotoxin B) with the crude venom. These positive reactions do not conclusively prove that there are PLA_2 toxins in the venom, but they do suggest that the structure of the molecule is antigenically similar to that of other PLA_2 toxins. Due to the specificity of antibody-antigen reactions, these results are highly suggestive. This evidence when coupled with the presence of lesions identical to those noted for other PLA_2 toxins lends credence to the proposal that this venom contains PLA_2 myotoxins. These data also reveal another interesting point that also can be supplemented by the histological evaluation of the lesions produced by crude venom. The immunoblot analysis of crude *S. m. barbouri*

venom with antiserum against myotoxin *a* , a small, basic myotoxin from the venom of *Crotalus viridis viridis* known to induce a characteristic vacuolation of the sarcoplasmic reticulum *in vivo* in mouse muscle, yielded no antigenic recognition. This fact along with the lack of these types of lesions in the damaged muscle cells suggests that this toxin is not present in *Sistrurus miliarus barbouri* crude venom.

Further research areas that must be investigated should include first the fractionation of the crude venom and isolation of specific toxic components. Upon isolation and characterization of these toxins, it will become possible to more accurately determine the structure/function relationships of these toxins and to relate these findings to those of other snake venoms. Predicting from what was observed in these studies, it could be suggested that there exists one or several toxins with structures similar to the PLA_2 types. Whether or not these toxins have enzymatic activity could be then determined and mechanisms further investigated. A comprehensive understanding of the mechanisms and toxins involved in tissue damage caused by this and other rattlesnake venoms will further the effective treatment of these occurrences. Certainly, the study of the pathogenesis of local tissue damage induced by venoms from snakes within the *Sistrurus* genus has proven to be an interesting and important area of study deserving of much more study.

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